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The Identification and Isolation of Human Alarm Pheromones

PI: LR Mujica-Parodi, Ph.D.
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Progress Report: Phase 0
April 30, 2006

Using funds from a DARPA-sponsored "seedling" grant, we have been successful in resolving the primary technical difficulties involved in this proposal, as well as obtaining preliminary data that informs our approach.

I. Optimization of Sample Collection

For the study design proposed for Phase 0, the putative alarm pheromone needed to be collected via axillary sweat samples obtained during reference (physical exercise) and arousal (skydive) conditions.

The design of the sweat pad had certain critical requirements. It needed to promote an efficient collection of axillary sweat, could not interfere with the subject's performance during the collection, and had to prevent evaporation of volatile components, the latter of particular concern during the skydive, where subjects reach vertical speeds of 165mph during one full minute of freefall. Importantly, it was necessary that the collection method not discrimininate between apocrine and eccrine secretions. Finally, the sorption material had to be compatible with the further chemical analysis, and thus rigorously minimize any contamination that might produce artifact in the GC-MS.

Unfortunately, it quickly became apparent that there was no commercially available product fulfilling all the mentioned requirements, which meant that we needed to first carefully design a pad before data collection could begin.

The optimization of the design was primarily focused on efficient sorption of secreted sweat. The sorption material was combined with a materials preventing evaporation of volatile components that were of a particular for identification of the target compounds.

The adsorption materials initially considered included nonspecific sorbents like cotton gauze, filtration paper, amphiphilic sorbent like polyvinyl alcohol or ethyl-vinyl acetate, and hybrophobic sorbent like PDMS polymer.

Filtration paper and cotton gauze were used as a low selectivity but a high capacity adsorbents, able to retain non-selectively large amount of sweat.

Filtration paper was excluded, due to the insufficient mechanical stability of the wet material and low flexibility of the material when dry. Cotton gauze showed similar sorption capacity and efficiency combined with a significantly better mechanical properties. Cotton gauze was therefore used as the primary absorbent for further experiments.

We also attempted to improve the sorption capacity and selectivity of the sweat adsorption material by introducing an additional sorption layers containing an amphiphilic or hydrophobic materials. This approach however did not significantly improved sorption capacity and selectivity. Cotton gauze likely prevented access of sweat to amphiphilc or hydrophobic layer, and sorption of sweat components by these layers was inefficient. This fact was confirmed by GC-MS, where the detection of the absorbed components was problematic.

Therefore, initially simple sterile woven cotton gauze sponges were used as a main sorption material during the first 20 experiments. Axillary sweat was collected with the use of cotton

sponges (2x2", Dukal, USA) taped in the underarm with waterproof adhesive tape (2", HYTAPE, USA). The waterproof tape promotes stable fixation of the gauze pad during exercise and skydive and also prevents evaporation of volatile components.

Commercially available gauze sponges contain quite a few small molecule additives that have to be removed before use. We therefore pre-extracted the sterile gauze pads multiple times in Ethanol (99.9%, HPLC grade purity, Sigma-Aldrich, USA) and Hexane (HPLC grade purity, Sigma-Aldrich, USA) and dried them before use. We confirmed the cleanness of the pre-extracted gauze pads by GC-MS.

To prevent a possible contamination of the cotton sponge with the tape glue, an additional thin Mylar isolation layer, previously extracted in Ethanol (99.9%, HPLC grade purity, Sigma-Aldrich, USA), was used.

Figure 1: Because there is no commercially available product suitable for the sweat collection, we developed our own sweat pads that were optimized for both the physical rigors associated with the skydive, as well as the purity required for GC-MS.

Subjects employ a wide range of motion during skydiving, which could dislodge the sweat pads or permit evaporation. Therefore, the pads were additionally fixed at place with the use of custom-made neoprene garments, each constructed in six different sizes to accommodate a close fit for a wide range of body-types. The garments ensured that we were able to minimize sample loss to evaporation during the fall while maintaining the wearer's comfort.

II. Development of the Extraction Protocol

As a general strategy we have employed two consecutive solvent extractions before GC-MS analysis. The idea behind this procedure is to extract our target substances into progressively more hydrophobic solvents (water → primary extraction solvent → secondary extraction solvent) while concentrating them.

After the sweat collection, the pads were extracted with 15 ml of the primary extraction solvent. For this purpose we compared extraction efficiency of Ethanol and Ethyl acetate. Consecutive GC analysis of the exercise sweat extract revealed only a limited extraction efficiency when Ethyl Acetate was used as a primary extraction solvent. A number of identifiable components were significantly lower compared to the Ethanolic extraction (Fig 2). For this reason, Ethanol was selected as a primary extraction solvent for further experiments.

For secondary extraction we developed two alternative procedures:
1) We evaporated 1 ml of the ethanolic extract to dryness using a speed-vac system. We then re-dissolved the sediment in 10μl of hexane that was then injected into the GC-MS. This procedure is very efficient in concentrating the sample but may have resulted in the loss of volatiles during the drying process.

2) For this reason we developed a secondary extraction procedure that relies on solvent partitioning. We mixed 2ml of ethanolic extract with 8ml of Millipore water and added 0.5ml of hexane that was doped with 2ng/ml chrysene as a tracer molecule. After vigorous shaking the mixture is centrifuged at 10,000 rpm to separate the ethanol-water and hexane phases. We then carefully aspirate 350μl of the hexane phase using a Hamilton syringe. After that, we concentrate the sample by blowing down the hexane with argon until about 30μl of hexane extract remains. The chrysene serves as an internal standard to the original concentration before evaporation allowing us to measure absolute amounts of the sweat components. In the future we will refine this method further to be applied to HPLC fractions also.

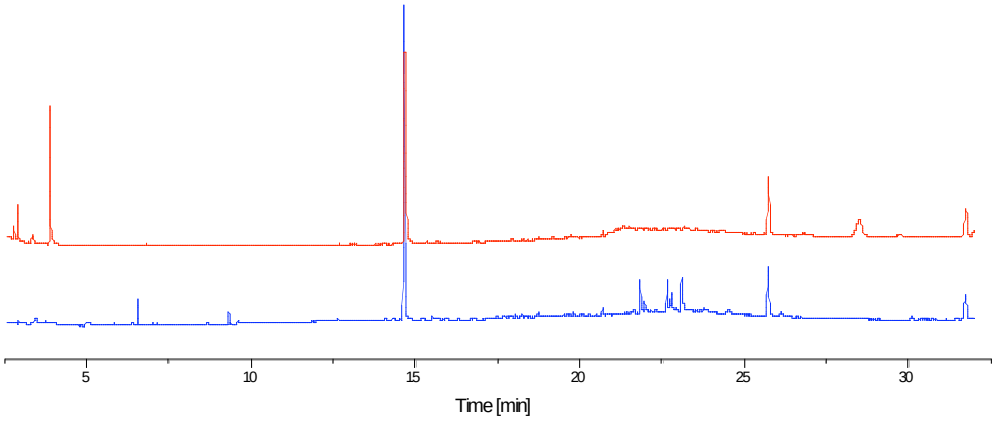

Figure 2: Comparison of the extraction efficiency of the Ethanol (-) and Ethyl-acetate (-) as an extraction solvents. Sweat adsorption pads were extracted in 15 ml of extraction solvent, concentrated by vacuum centrifugation and dry mass equivalent to 3 ml of the sweat extract was re-dissolved in 50 ml of cyclohexane and analyze by GC.

III. Preliminary Identification of Steroids of Interest in Human Fear Sweat Using Skydiving Protocol

During Phase 0, we collected sweat, urine, blood, saliva, ECG, respiration, and self-report measures in 20 subjects (n=11 males and n=9 females) before, during, and immediately following their first-time tandem skydive, as well as before, during, and immediately following their running on a treadmill for the same period of time. Measurements between the test (skydive) and control (exercise) conditions were made on consecutive days, each experiment precisely matched to the minute between subjects and between conditions to prevent diurnal confounds. The sweat and urine were analyzed using GC-MS. The ECG, spirometry, blood, and measures were used to confirm levels of arousal via changes between conditions in heart rate, respiration, cortisol, DHEA, epinephrine, and neuropeptide-Y. Emotional states were monitored using brief standardized questionnaires that serially assessed calmness, anxiety, aggression, euphoria, cognitive clarity etc. These were later reviewed with the tandem-master, who provided insight into the subject's behavior immediately preceding the jump. We additionally administered cognitive tests using PDA's attached to the subject's forearm before, during, and after test and control conditions in order to assess the effects of arousal on cognition. Our results confirm both that this collection paradigm is controlled and reproducible (as shown by altimeter data, Fig 3) as well as successful in inducing acute emotional stress (Table 1).

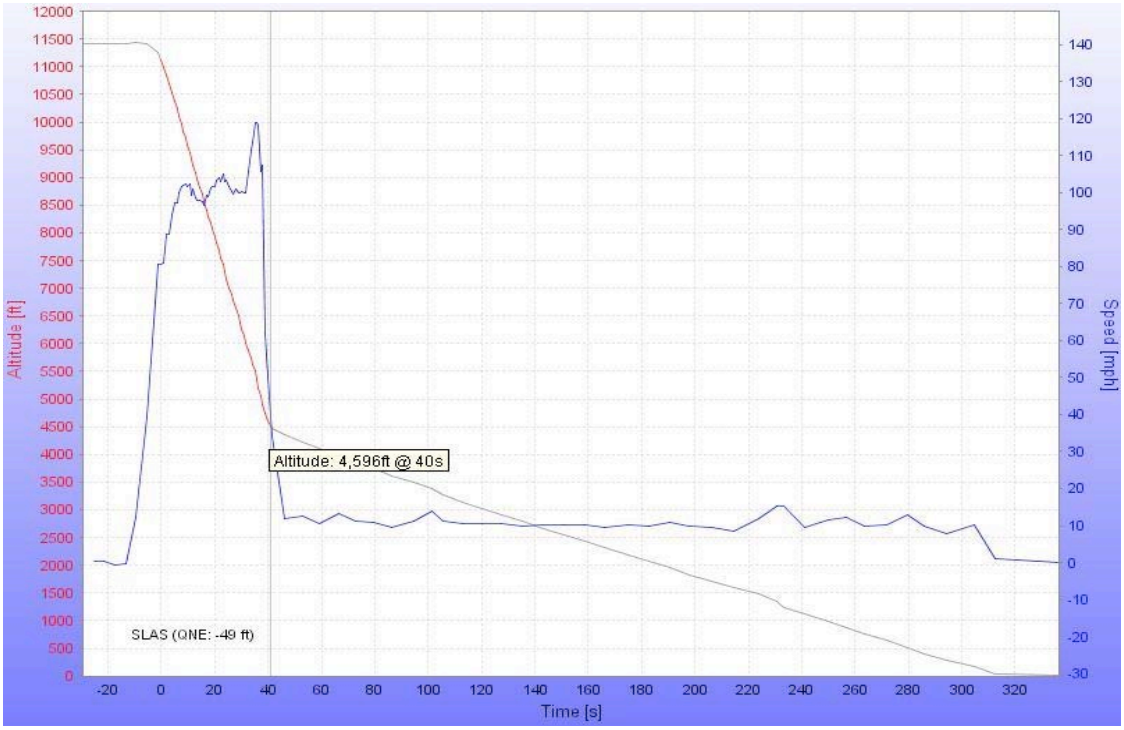

Figure 3: Altimeter Data Confirm that Skydives Produce a Reliable Protocol for Testing

As shown in Table 1, while both the Test (skydive) and Control (exercise) conditions induce autonomic changes associated with non-specific physiological arousal (increase in HR), only the Test condition produced the emotional stress component, as demonstrated by an increase in cortisol (stress-hormone). This would suggest that any biochemical differences associated with the two conditions are the result specifically of emotional, and not physical, stress.

Measure (n=20)	Baseline	SD	Jump	SD	F	p
BEFORE VS. AFTER (DIFFERENCE IN TIME: 20 MINUTES)						
TEST CONDITION (SKYDIVE)						
LF-NU (Sympathetic ANS)	.742	.035	.847	.022	21.581	0.001
HF-NU (Parasympathetic ANS)	.258	.035	.153	.022	21.581	0.001
Heart Rate	96.643	4.318	122.615	4.944	24.709	0.000
Cortisol	13.715	1.997	22.891	3.358	8.059	0.019
CONTROL CONDITION (EXERCISE)						
LF-NU (Sympathetic ANS)	.463	.030	.279	.022	24.748	0.000
HF-NU (Parasympathetic ANS)	.150	.019	.125	.025	.572	.461
Heart Rate	82.324	3.230	162.224	4.137	333.470	0.000
Cortisol	18.727	2.268	14.338	1.303	3.610	0.080
TEST VS. CONTROL (IDENTICAL TIMES TO CONTROL FOR POTENTIAL DIURNAL VARIANCE)						
Heart Rate	162.754	4.276	122.615	4.944	37.551	0.000
Cortisol	16.362	1.997	22.808	2.459	5.150	0.037

Table 1: First-time Skydive Provides a Reliable Acute Stressor

Our analytical work using GC-MS, investigating the differences between the two conditions, indicate that there were significant and trend increases in components of the emotional stress sweat as compared to the physical stress sweat. These were predominantly in the m/z = 229, 272, 270, and 231 regions (Fig 4). Each individual's data was normalized to his or her cholesterol peak (m/z = 386.5), to avoid confounds due to the amount of sweat secreted.

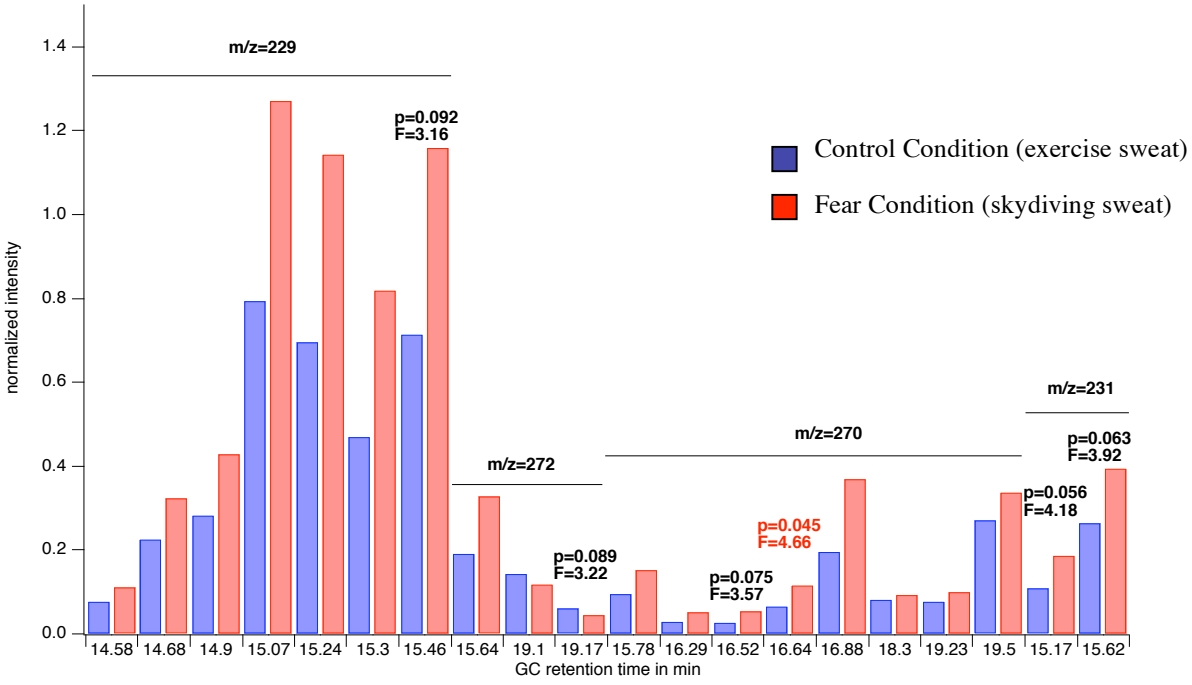

Figure 4: GC-MS Shows Relevant Candidates of Interest Among Steroids Increased During Fear vs. Exercise (sex=covariate)

Interestingly, differences were only observable when using sex as covariate, since there was a strong sex differential within the responses. Consistent with the general pheromone literature, in which men most strongly emit the chemosensory signals while women most strongly detect them, we found that for most of the observed compounds (RT=15.46, 16.52, 16.64, 15.17, and 15.62), men showed an increase in the compound emission during acute emotional stress, while women showed either no change or a decrease in emission of this compound (Fig 5). These differences are not due to different volumes of sweat produced between men and women, since all samples were normalized to volume using the cholesterol peak (m/z = 386.5). Also, there were no significant differences between the amounts of these compounds during the control condition, indicating that the compounds of interest are not simply sex-dependent; rather, the differential is specific to emotional stress.

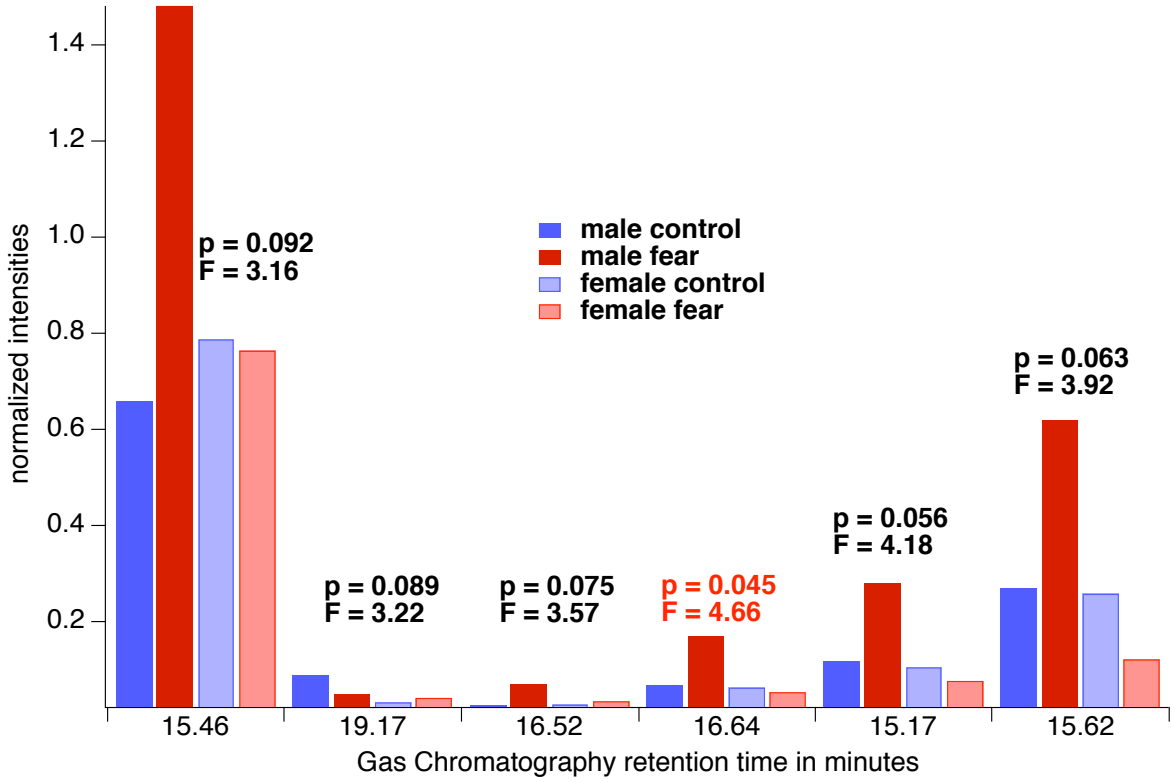

Figure 5: Equivalent Steroids of Interest Between Males and Females During Control Condition Show Differential Sex Response Between Males and Females During Fear

These sex-dependent results have important experimental implications for our protocol, since they imply that either we will need to test only men, or that we will need to essentially double our data collection (from n=20/year to n=40/year) for the olfactometry portion of the study if only men will produce significant quantities of the compounds of interest. We choose to do the latter, in spite of the tight schedule (two subjects per week rather than one), so that we may continue to investigate the role of sex differences in a larger sample

Based upon these results, our preliminary analytical work using GC-MS indicates several candidate compounds similar to (Fig 6).

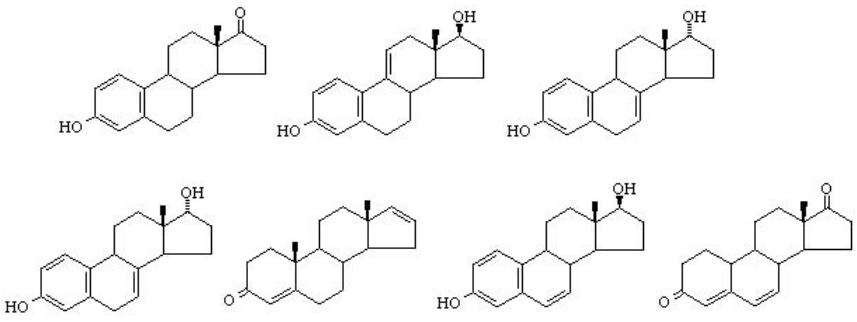

Figure 6: Preliminary Candidate Compounds As Suggested by GC-MS Analysis of Fear vs. Control Sweat

From the distributions of the molecular fragments for the considered retention times we can tell that the putative fear pheromones are derived from the steroid families of estrogens and androgens of 270 and 272 molecular weight. None of the steroid standards in the literature matched the retention times and the fragment distribution. This may indicated that the putative fear pheromone is derivatized in a thus far unknown fashion. The fact that our target peaks elude earlier than most steroid standards may point towards a derivatization with hydrophobic moieties.

III. Olfactometry

Design and Testing of Olfactometer

While the selective secretion of these compounds during fear is highly suggestive, in order to determine whether the compounds we isolated are alarm pheromones, it is important to test whether they trigger arousal in other humans using neural (fMRI) and autonomic measurements. A critical first step in doing so is to ensure the accurate and reliable presentation of olfactory stimuli. Unfortunately, there are only a few MRI-compatible olfactometers commercially available, and each of these uses delivery systems that are incompatible with our extracted sweat samples. Therefore, we first needed to build and test two MR-compatible (non-ferrous) olfactometers. Given the budget restrictions of Phase 0, we elected to use the Lorig-design (http://psych.wlu.edu/cnl/olfactometer_construction.htm). A schematic of this equipment is shown in Figure 7. This design allows switching between six samples without change in flow velocity to the nostril (approx. 1.5 l/min).

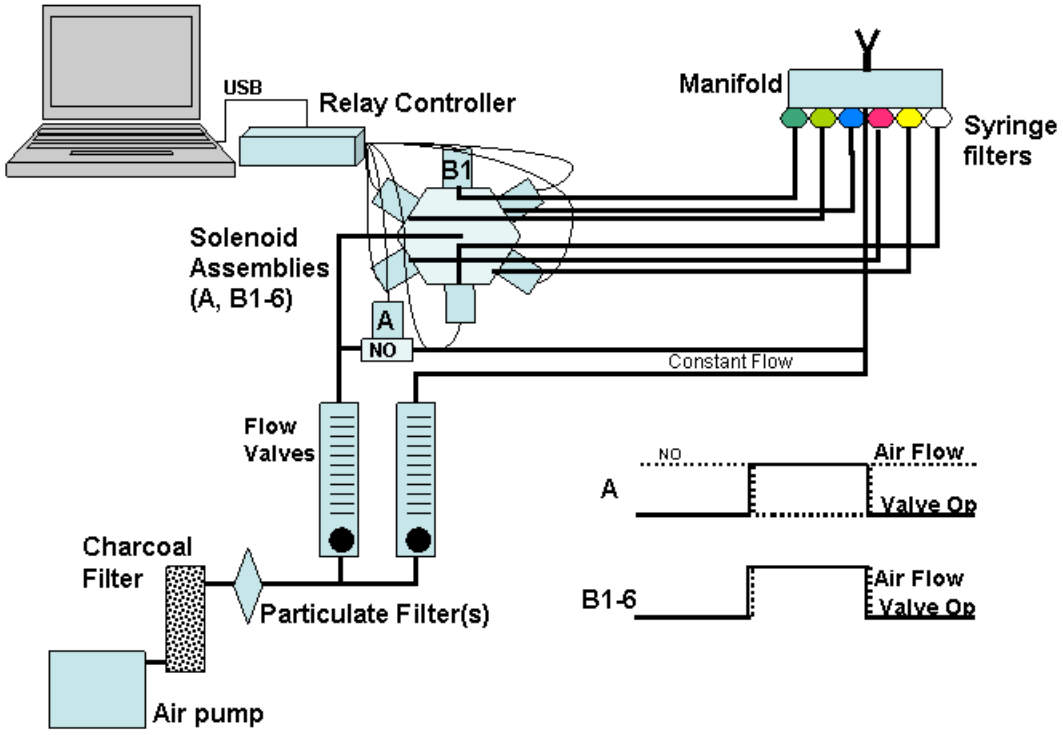

Fig 7: Schematic of the Lorig olfactometer that will be used in this study. The olfactometer is designed to exhibit no change of air velocity while switching between samples (http://psych.wlu.edu/cnl/olfactometer_construction.htm).

In the original design, odor-containing liquid (e.g. mineral oil containing a frangrance) is pipetted into syringe filters that plug into the manifold. The odor-saturated air in the syringe filter assembly is delivered into the nostril by airflow through the filter. To avoid leakage of odor into the manifold each syringe filer is isolated from the air channel by a one-way-valve that only opens when air is pushed through the filter.

In order to use this olfactometer for our study we needed to modify the sample delivery, since the delivery system was designed for common odors and fragrances that are abundantly available. In our case, we expect the fear pheromones to be semi-volatiles, making the delivery more challenging. For more efficient delivery we have designed a holder for glass fiber filters. Before use these glass fiber filters are immersed into hexane extracts with subsequent cold evaporation of hexane in a freeze dryer. By this procedure, the active semi-volatiles coat the large surface area of the glass fiber filters and allow efficient volatilization for use in an olfactometer. To avoid leakage into the manifold, as well as leakage of the semi-volatiles back towards the air-pump we have braced the filter assemblies with two one-way valves to maximize the odor concentration in the filter assembly air. When air is directed through the filter assembly the semi-volatiles can be efficiently delivered to the nostril.

Because the extracted sweat samples are so difficult to obtain, we have first thoroughly tested the olfactometers with common odors. By placing the sample containing manifold close to the nostrils, we have been able to achieve a rapid sharp delivery of odors. After experimentation, we

determined a 2 seconds delay between switching the valve and the subject perceiving the odor. As seen in figure 8, the olfactometer can easily be used in combination with an auditory startle experiment, to determine if the compounds increase the startle reflex.

Fig. 8: Olfactometer manifold combined with an auditory startle experiment. The manifold containing the odors is placed close to the subjects nose to allow rapid and sharp delivery.

In this experiment, already tested with common odors to ensure a smooth interface between the computerized components of the stimulus delivery and physiological measurements, the subject has three 4 mm shielded electrodes placed on his/her face to record startle response: one is placed directly under the dominant eye (aligned with the iris as the subject looks directly ahead), one is placed to the side of the dominant eye, 1mm from the outside corner of the eye, and one is placed on the center subject's forehead (ground electrode). Subjects additionally have skin conductance (SC) and EKG electrodes attached, as well as a respiratory belt. Velcro SC electrodes are attached to the underside of the second and third fingers of the subject's non-dominant hand. Shielded EKG electrodes are attached to the subject's chest: one directly above the heart, and one at each side of the subject's waist. The respiratory belt are placed directly over the sternum, and tightened while the subject exhales. Physiological data from all electrodes and the respiratory belt are collected and recorded via the Biopac Systems MP150 module (Fig.9). Auditory stimuli are administered via Bose Acoustic Noise-Cancelling headphones.

The task consists of thirty-one blocks (duration of 15 seconds) alternating between rest and test conditions. During the test blocks one of three chemosensory stimuli is presented (aversive, pleasant or neutral; or with the sweat extracts: fear sweat, exercise sweat, and room air) simultaneously with acoustic startle probes (95 dB for 50ms) with an onset time of 7.5 seconds. During the entire task subjects are in a completely dark room and are asked to focus on an orienting cross (1.5 x 1.5 inches white cross-hairs), which is presented on a 47" plasma screen.

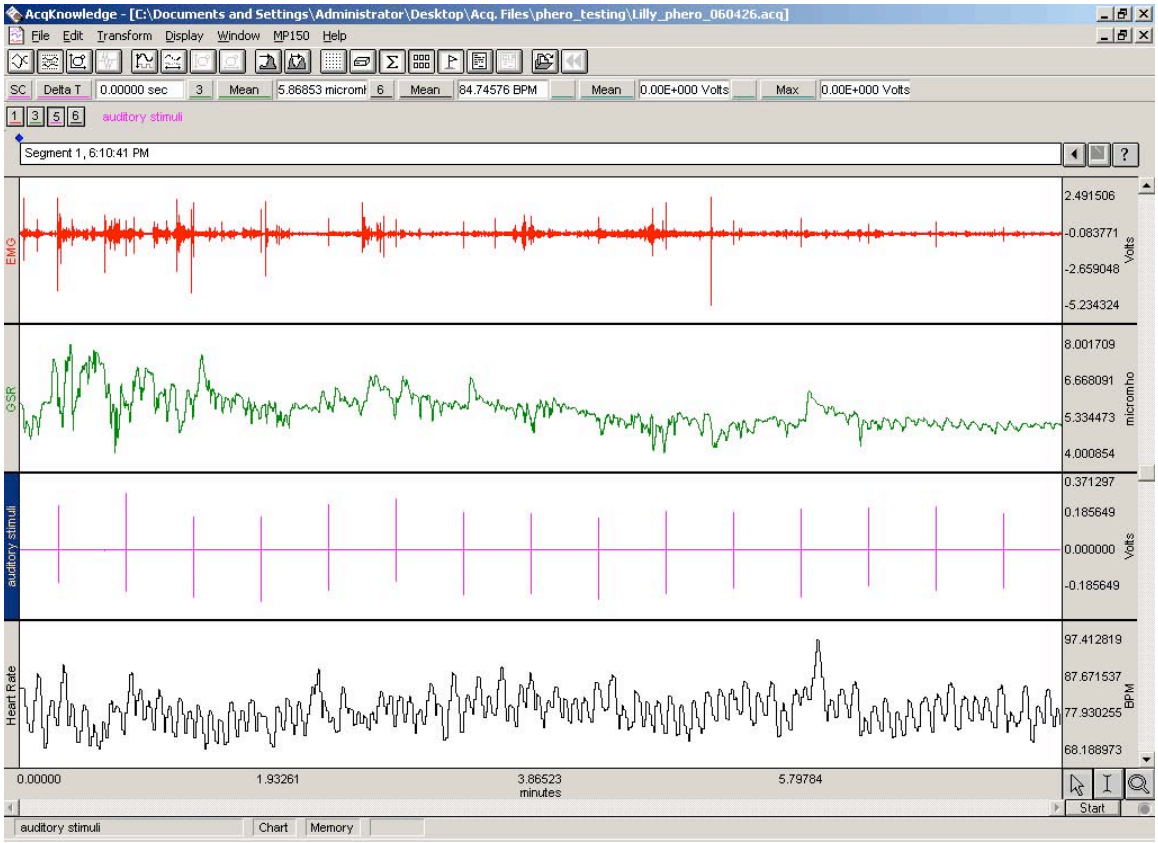

Fig 9: Physiological measurements obtained during preliminary olfactometry experiment using common odors. The identical experiment is now ready for use with sweat extracts.

An equivalent setup, which includes both olfactometer and physiological testing equipment, has been installed in our 3T MRI. To determine the optimal field strength for research on a putative alarm pheromone, which we hypothesize to affect the limbic regions, we performed preliminary testing (n=6) of a known aversive task (negative facial stimuli) on the 1.5T and 3T MRI at Stony Brook University Hospital, and the 4T MRI at Brookhaven National Laboratories. Signal to noise ratio values for the orbital frontal and amygdala regions were calculated by taking the ratio of the mean signal value in a region of interest (ROI) drawn in each of these regions and the standard deviation for a ROI placed outside the brain in the image background. Signal values in amygdala and orbital frontal regions were also compared to the signal value in a ROI placed in the cerebellum. Comparison of these values demonstrated that the 3T MRI provides the optimal signal-to-noise ratio for our protocol (Table 2) with a minimum of susceptibility artifacts in the orbital frontal and amygdala regions (Table 3), which are most vulnerable to these distortions. Therefore, we will conduct our proposed olfactometry research on the Stony Brook University Hospital's 3T MRI.

ROI	1.5T	3T	4T
Left amygdala	29.99	86.32	130.76
Right amygdala	30.80	70.42	119.06
Prefrontal cortex	27.29	67.27	156.02
Cerebellum	26.80	58.70	143.65

Table 2: Comparison of Signal to noise ratio (SNR) values for ROIs with 1.5T, 3T, and 4T MRI.

ROI	1.5T	3T	4T
Left amygdala	1.118	1.469	0.910
Right amygdala	1.151	1.199	0.828
Prefrontal cortex	1.017	1.145	1.086

Table 3: Comparison of Signal Drop-Out for ROIs with 1.5T, 3T, and 4T MRI

Given the thoroughness of this initial developmental work in Phase 0, we are now in a position to commence an iterative process that will allow us to "zero in" on the exact chemical composition of the putative alarm pheromone, as proposed in our Phase 1 application. This process involves not only comparing the candidate with known steroidal standards, but also using HPLC fractionation to progressively separate out individual components, then using these fractionated samples as olfactory stimuli to assess neurobiological responses to them.